Expression of Neuronal and Inducible Nitric Oxide Synthase Isoforms and Generation of Protein Nitrotyrosine in Rat Brain Following Hypobaric Hypoxia

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Summary

The expression of neuronal and inducible nitric oxide synthase (nNOS and iNOS) and nitrotyrosine immunoreactivities in the cerebral cortex, caudate putamen, islands of Calleja, hippocampus, superior colliculus and cerebellum of rats submitted to hypobaric hypoxia equivalent to an altitude of 30,000 feet (9144 m; barometric pressure 230.4 Torr) were analyzed and semiquantitatively assessed by means of light microscopic immunocytochemistry and Western blotting using specific polyclonal antibodies. Changes in the expression of these components were directly related to the reduction of barometric pressure, time of exposure to hypobaric hypoxia and the post-hypoxic recovery period. After exposure to hypobaric hypoxia for 8 h and a recovery period of 24 h, an intense vasodilatation was also demonstrated in blood vessels throughout the brain and especially in blood vessels of the hypothalamic magnocellular accessory group. Animals treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME) did not show changes in nNOS and iNOS expression but displayed decreased nitrotyrosine immunoreactivity. The anatomical and biochemical modifications following 4 or 8 h of hypobaric hypoxia demonstrated by this study may be related to some of the psychological changes described after human exposure to high altitude.

Introduction

Few experimental studies on animals have been carried out on the biological mechanisms underlying the debilitating neuropsychiatric disorders that may affect unacclimatized individuals when exposed to the hypobaric hypoxic conditions of high altitude for periods ranging from several hours to days (Carson et al., 1969; Hackett and Rennie, 1976; Hultgren, 1979). These disorders, characterized by symptoms such as headache, insomnia, irritability, depression and impaired sensory, memory and cognitive functions (Carson et al., 1969), are clearly related to functional changes in the central nervous system (CNS) and Forster et al. (1975) demonstrated increased cerebral electrical activity by electroencephalography (EEG) and a reduced visual evoked response signal.

The severity and duration of these symptoms vary with the exposed individual's condition, the altitude and rate of ascent (Hansen et al., 1967), and symptoms sometimes persist after returning to lower altitudes (Ryn, 1971; Sharma et al., 1975; Sharma and Malhotra, 1976; Townes et al., 1984; West and Lahiri, 1984; West, 1986; Cavaletti et al., 1987, 1990; Oelz and Regard, 1988; Regard et al., 1989; Cavaletti and Tredici, 1992; Shukitt-Hale et al., 1994). Cavaletti et al. (1987) reported a decrease in memory performance tested at sea level 75 days after a climb without supplementary oxygen to 7075 m (23,212 ft.). This was confirmed by Kassirer and Such (1989), who reported that certain neurologic symptoms (headache and taste dysfunction) persisted 15 days after an accidental exposure to hypoxia in a pressure chamber, simulating an altitude of 7620 m (25,000 ft.). The hippocampus, one of the cerebral regions most vulnerable to hypoxia (Arregui et al., 1991; Xun et al., 1991; Shukitt-Hale et al., 1996), also appears to show changes related to a definite impairment of mental ability (Haldane and Priestley, 1935).

It is generally accepted that psychomotor performance is not impaired at altitudes below about 3048 m (10,000 ft.) (Figarola and Billings, 1966; Pearson and Neal, 1970; Ernsting, 1978), but many neuropsychologic functions deteriorate as altitude increases above 4000 m (13,123 ft.) (Fine and Kobrick, 1978). However, there is evidence that learning of complex mental tasks is slower at pressures equivalent to an altitude of 2438 m (8000 ft.) (Denison et al., 1966), or even at altitudes as low as 1829 to 2438 m (6000 to 8000 ft.) (McFarland, 1971), than at sea level. Thus it appears that even at the cabin altitudes of today's commercial aircraft (2500 m or 8200 ft.) sensitive psychometric texts can detect minor degrees of impairment. After a prolonged stay at high altitude (3500 m or 11,483 ft.) changes in autonomic nervous activity were also reported by Malhotra and Mathew (1974), in the form of sympathetic and parasympathetic hyperactivity. Systolic and diastolic blood pressures and the mean pulse rate also increased at 4200 m (13,780 ft) (Forster, 1985).

The changes in the central and peripheral nervous systems consequent on exposure to hypobaric hypoxia may be mediated by the endogenous generation of nitric oxide (NO), which as an intercellular messenger and potent vasodilator is directly involved in various pathophysiologic mechanisms. NO is synthesized from L-arginine in the mammalian

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brain (Knowles et al., 1989; Bredt et al., 1990, 1991) as well as in invertebrate neural structures (Elphick et al., 1993; Martinez et al., 1994) by the enzyme nitric oxide synthase (NOS), which has been the subject of extensive study (for reviews, see Moncada and Higgs, 1993; Moncada et al., 1991; Rodrigo et al., 1994, 1997, 2000).

There is now evidence that at least three forms of NOS can be distinguished, two constitutive isoforms (cNOS) and an inducible isoform (iNOS) (Forstermann et al., 1991; Moncada et al., 1991). The cNOS isoform present in vascular endothelial cells is known as endothelial NOS (eNOS), and that present in the central and peripheral nervous system is known as neuronal NOS (nNOS) (Bredt et al., 1990, Moncada et al., 1991). The latter has been purified from rat brain and cerebellum (Bredt and Snyder, 1990; Knowles et al., 1990) and cloned from rat (Bredt et al., 1991) and human brains (Nakane et al., 1993). This isoform has been described as a soluble homodimer of 155 kDa (Bredt and Snyder, 1990; Schmidt and Murad, 1991), with a sequence similarity to cytochrome P-450 reductase at the carboxy-terminal end (Bredt et al., 1991). The molecule has recognition sites for nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and calmodulin (Bredt et al., 1991). This form of the enzyme is cytosolic and totally dependent on activation by Ca^{2+}/calmodulin, using L-arginine as substrate and NADPH as cofactor (Knowles et al., 1989; Bredt and Snyder, 1990, 1992; Garthwaite, 1991; Moncada et al., 1991). Biochemical measurements in different regions of the brain have shown that the highest concentration of NOS is found in the cerebellum, followed by the hypothalamus, midbrain, striatum and hippocampus, with the lowest activity in the medulla oblongata (Forstermann et al., 1990).

The inducible isoform of NOS has been purified from the cytosol of activated murine macrophages (Hevel et al., 1991) and shown to have a molecular mass of 135 kDa. It has been identified in mast cells, lymphocytes, neutrophils, hepatocytes, vascular smooth muscle cells, mesangial cells, endothelial cells, neurons and in various tumors (Busse and Mulsch, 1990; Marsden and Ballermann, 1990; Salvemini et al., 1990; Gross et al., 1991; Moncada et al., 1991; Moro et al., 1998). In rodent macrophage cell lines (RAW 264,7 and J774) the synthesis of iNOS is dependent on induction by lipopolysaccharide (LPS) and cytokines (Marletta et al., 1988). The enzyme also requires NADPH, FAD and FMN and to varying degrees, tetrahydrobiopterin BH_4 and glutathione for full activity (Stuehr et al., 1990; Hevel et al., 1991). The induction of iNOS can be inhibited by glucocorticoids (Moncada et al., 1991). Murine iNOS was cloned (Lyons et al., 1992; Xie, 1992; Lowenstein et al., 1993) and found to be distinct from the two known constitutive isoenzymes. Human iNOS has also been cloned, both from chondrocytes (Charles et al., 1993) and hepatocytes (Geller et al., 1993).

The neurotoxic effects of NO is mediated by formation of peroxynitrite and other highly reactive nitrogen species (Beckman, 1996). Peroxynitrite is capable of nitrating tyrosine residues in tissue proteins, and the nitrotyrosine formed over a period of time can be used as a marker of peroxynitrite production (Beckman, 1996; Beckman et al., 1992). Immunohistochemical studies have revealed that nitration of tyrosine residues takes place in various organs after ischemia and in other pathological conditions (Ischiropoulos et al., 1992; Wang and Zweier, 1996)., e.g. in the brains of patients with Alzheimer's disease (Smith et al., 1998) or in the cerebral cortex of the aging rats (Uttenthal et al., 1998).

Pharmacological agents can reduce NO production or prevent its biological effects in a variety of ways, e.g. by inhibiting cellular L-arginine uptake, by reducing the cellular availability of necessary cofactors, by inhibiting the NOS enzymes, by scavenging NO once formed, or by inhibiting the cellular mechanisms leading to the induction of a particular NOS isoform (Moncada et al., 1997). One of the most frequently used agents, N^{ω}-nitro-L-arginine methyl ester (L-NAME), is an unselective inhibitor of NOS isoforms that has also been reported to reduce glutamate efflux, a phenomenon that was considered to contribute to the reduction of infarct size after focal ischemia (Buisson et al., 1992, 1993). However, the importance the latter effect is in doubt, as animals treated with L-NAME have been reported to show increased, decreased or unchanged glutamate efflux in comparison with controls (Zhao et al., 1999).

The aim of the present study was to investigate the effects of exposing rats to acute hypobaric hypoxia at 230.4 Torr (simulating an altitude of 30,000 ft. or 9144 m) for periods of 15 min to 8 h on the expression and localization of nNOS and iNOS in the CNS and to use nitrotyrosine in proteins as a marker for the production of reactive nitrogen species. Different brain cortical areas, the caudate putamen, the islands of Calleja, the hippocampus, superior colliculus and cerebellum were analyzed by immunocytochemistry and Western blotting using specific rabbit polyclonal antibodies against nNOS, iNOS and nitrotyrosine. We also investigated whether such changes could be suppressed by concurrent L-NAME administration, which might exert a protective effect on the brain under these conditions.

Material and methods

The study was performed on 60 young male albino Wistar rats (220-350 g body weight), which were subjected to periods of simulated high altitude in a hypobaric chamber (Environmental Tectonics Corporation International (EETC) type 10M. This chamber can simulate a variety of atmospheric conditions by reducing the ambient barometric pressure in combination with a precise control of temperature and relative humidity. The chamber is continuously purged with fresh air, replacing oxygen consumed by the rats and removing carbon dioxide. Altitude simulation is achieved by means of a vacuum pump to reduce pressure in the chambers.

Experimental procedures

Groups of 10 rats were exposed to a simulated altitude of 30,000 ft. (9144 m; 230.4 Torr) for 15 min, 45 min, 4 h or 8 hours, a control group being kept under normobaric, normoxic and standard conditions of light and temperature with free access to water and commercial rat chow. A further group of 10 rats were pretreated with 1.5 mM L-NAME (0.4 mg/ml) added to the drinking water (Cellek et al., 1999) for five days; six of these were submitted to the same simulated altitude for 8 h, the remaining four serving as L-NAME controls. This dose of L-NAME is higher than the 5 mg/kg dose considered to inhibit NO synthase activity (Navarro et al., 1994).

After exposure to simulated altitude, half the animals in each group were immediately perfused for either immunocytochemistry or Western blotting and the other half were perfused after a 24-h post-hypoxic recovery period under normobaric, normoxic conditions. L-NAME administration was continued throughout the recovery period in the L-NAME pretreated rats. Animals were then deeply anesthetized with pentobarbital (25 mg/kg i.p.) and perfused with 0.9% saline through the left ventricle via a blunt cannula (Minoject, St. Louis, MO, USA) connected to a peristaltic pump (Microtube pump MP3, Eyela, Tokyo, Japan). For Western blotting, perfusion was stopped after blood had been rinsed from the vasculature with 50-100 ml saline; the brain was then removed for extraction of proteins. For immunocytochemistry, perfusion was continued with 500 ml fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, cut into blocks and postfixed for a further 4 h in the same fixative solution at room temperature. The blocks were then rinsed and cryoprotected by immersion overnight at 4°C in 0.1 M PB containing 30% sucrose, with continuous stirring.

Immunocytochemistry

Serial frontal frozen sections, 40 µm thick, were cut with a Leitz sledge microtome. Free-floating sections were processed by the avidin-biotin peroxidase complex (Rodrigo et al., 1994) to visualize nNOS, iNOS and nitrotyrosine immunoreactive sites. All sections were incubated for 30 minutes in PBS containing 3% normal goat serum (ICN Biochemicals, Barcelona, Spain) and 0.2 % Triton X-100, and then separately with nNOS, iNOS and nitrotyrosine antisera, diluted 1:3000, 1:5000 and 1:1000, respectively, in PBS/Triton X-100, overnight at 4EC. After several washes in PBS, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin for 1 h. After washing, the sections were incubated with peroxidase-linked ABC (Vector Laboratories. Burlingame, CA, USA) for 90 minutes. The peroxidase activity was demonstrated by the nickel-enhanced diaminobenzidine procedure (Rodrigo et al., 1994).

Immunocytochemical controls. Control procedures were carried out on sections from two animals. No immunolabeling was observed when the primary antibodies were omitted or replaced with an equivalent concentration of preimmune and normal rabbit serum. The specificity of the nNOS antiserum was demonstrated by incubating the tissue sections with the primary antiserum dilution preabsorbed (overnight at 4°C) with 2 µg/ml recombinant nNOS; specificity of the iNOS antiserum was demonstrated by preabsorption with the respective immunizing peptide at 10 nmol/ml. The specificity of the nitrotyrosine antiserum was demonstrated by preabsorption with free 3-nitrotyrosine at 100 nmol/ml. This procedure abolished immunostaining in all three cases.

Western blotting

Unfixed brains were homogenized (1:5 w/v) in 50mM HEPES buffer, pH 7.4, containing 100 mM KCl, 10mM $MgCl_2$, 10 mM NaH_2PO_4, and 0.33 mg/ml soybean trypsin inhibitor. All procedures were carried out at 4ºC. Homogenates were centrifuged at 105,000g for 1 h and the supernatant collected. Protein concentrations were determined by the method of Bradford (1976), using BSA as standard. Samples of supernatants containing 30 µg protein were heated to 95ºC for 3 min in 62 mM Tris-HCl buffer, pH 6.8, containing 2% w/v SDS, 10% v/v glycerol and 10mM 1,4-dithiothreitol. Proteins were separated by SDS-PAGE in 7.5% gels for nNOS and 12.5% gels for protein nitrotyrosine, in a Bio-Rad Mini-Protein II apparatus (Bio-Rad, Madrid, Spain), and were then electroblotted onto 0.2 mm polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) by means of a semidry transfer apparatus (Hoeffer, Pharmacia, Barcelona, Spain) at 1.5 mA per cm^2 of membrane for 2 h in 10 mM CAPS buffer (Sigma, Madrid, Spain), pH 9.4, to which 10% v/v methanol had been added. The membranes were blocked with 5% w/v dried defatted milk and 0.05% v/v Tween-20 in 10 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl. The membranes were then incubated with dilutions of the polyclonal nNOS, iNOS or nitrotyrosine antisera in blocking solution overnight at 4ºC. Bound antibody was revealed by means of an enhanced chemiluminescence kit (Amersham, Madrid, Spain), according to the manufacturer's instructions, and the membranes were scanned with a computer-assisted densitometer.

Antisera

Neuronal NOS. A rabbit polyclonal antiserum raised against full length recombinant rat brain nNOS (Riveros-Moreno et al., 1995) was a gift from Dr. V. Riveros-Moreno (Wellcome Research Laboratories, Beckenham, UK). This was characterized by immunocytochemistry and shown to react with neurons of different mammalian species, but only at low dilution with rat endothelium, its properties being closely similar to a polyclonal antiserum raised against purified rat brain NOS (Springall et al., 1992). It was further characterized in Western blots of rat liver and brain homogenates (Uttenthal et al., 1998). Rat endothelium was not stained by the antiserum at the dilution used in the present study.

Inducible NOS. Peptide PT387 (Ac-Cys-[residues 1131-1144]) from the C-terminus of the deduced sequence of murine iNOS (Moss et al., 1995) was custom synthesized by an automated 9-fluorenyl methoxycarbonyl (FMOC) solid-phase method (Peptide Therapeutics, Cambridge, UK), purified by reverse-phase high-pressure liquid chromatography (HPLC) and characterized by mass spectrometry. The peptide (1 mg) was conjugated via the Cys residue to 1 mg keyhole limpet hemocyanin (KLH) by the method of O'Sullivan et al. (1979) using maleimidobenzoic acid N-hydroxysuccinimide ester. One third of the conjugate was emulsified in an equal volume of Freund's complete adjuvant and used to immunize 4 New Zealand white rabbits by subcutaneous injection at multiple sites on the back. Four boosts of 1/6 of the conjugate emulsified in Freund's incomplete adjuvant were given by subcutaneous injection at monthly intervals thereafter, and the animals were bled from the marginal ear vein 14 days after each boost. Antibody titers were checked by enzyme-linked immunoassay in microwells coated with 5 μg/ml of a BSA conjugate of the same peptide (molar ratio peptide:protein 4:1) and characterized by Western blotting (Uttenthal et al., 1998).

Nitrotyrosine. Peroxynitrite was generated in aqueous solution as described by Blough and Zafiriou (1985) and stored in aliquots at -80°C. KLH (20 mg) was dissolved in 2 ml 25 mM sodium bicarbonate containing 1 nM EDTA and 1 mM ferric chloride. Peroxynitrite solution was thawed, its concentration determined by optical density at 302 nm, and added as a single aliquot to a calculated final concentration of 9 mM under continuous agitation at room temperature. The resulting solution (nitrated KLH) was dialyzed at 4EC for 24 hours against three changes of 1 l of phosphate-buffered saline, pH 7.4 (PBS). BSA (20 mg) was treated with peroxynitrite in parallel, following the same protocol. Two New Zealand white rabbits were each immunized at multiple subcutaneous sites with 2 mg nitrated KLH emulsified in a equal volume of Freund's complete adjuvant and boosted at monthly intervals with the same dose emulsified in Freund's incomplete adjuvant. Animals were bled as above and antibody titers were determined by EIA in microwells coated with nitrated BSA at 1μg/ml. The chosen antiserum showed 50% and 90% inhibition of antibody binding by 1.25 μM and 64 μM free 3-nitrotyrosine, respectively (Uttenthal et al., 1998).

Results

Immunocytochemistry

Control rats

nNOS immunoreactivity. Immunoreactive neurons were found at all levels of the cerebral cortex from rostral to caudal poles of the brain. These immunoreactive neurons, of fusiform, triangular and multipolar morphology, were distributed in layers II, III, IV and VI (Fig. 1A). They possessed a few long primary processes, which occasionally gave off long varicose collaterals (Fig. 1B). In general, all cortical areas showed discrete immunoreactive nerve fibers, which were especially densely distributed in layer I, where punctate immunoreactive fibers were also found.

The caudate putamen contained a large number of nNOS neurons which contributed processes to a varicose interstitial plexus between the unreactive fascicles crossing this region (Fig. 1C). The neurons were of medium size (15-20 μm) and showed a variable morphology, from fusiform to multipolar, with long, varicose and aspiny processes and few collaterals. The highest concentration of these immunoreactive neurons was found near the wall of the lateral ventricle.

The islands of Calleja contained numerous nNOS-immunoreactive granular cells. The insula magna at the mediodorsal border of the accumbens nucleus was limited medially by large immunoreactive neurons making up the vertical limb of the diagonal band of Broca. The other islands of Calleja are situated in the deep portion of the polymorph layer of the olfactory tubercle, limiting the ventral border of the accumbens nucleus (Fig. 1D). The granular cells of the islands of Calleja surrounded a dense network of small blood vessels. The clusters of granular cells in the area of the olfactory tubercle were included in a dense plexus of nNOS-immunoreactive varicose nerve fibers and large multipolar neurons of the polymorph layer of the olfactory tubercle.

The hippocampus showed scattered nNOS-immunoreactive neurons in all the areas studied (Fig. 2A). The dentate gyrus contained numerous nNOS neurons in the basal layer of the ectal and endal limbs of the granular cell layer. These neurons were multipolar with a large apical process and a few collaterals, which were mainly located between unreactive granular cells (Fig. 2B). Occasional nNOS neurons were found in the molecular layer of the dentate gyrus, mostly showing a fusiform morphology. The rostral sections of the hippocampus showed some nNOS neurons distributed in the pyramidal cell layer of the subiculum, and the CA1, CA2 and CA3 fields of Ammon's horn. These nNOS neurons (Fig. 2C) showed basal varicose processes in the stratum oriens and an apical dendrite composed of a single process with a few branches running through the stratum radiatum and forming a discrete tuft in the stratum lacunosum moleculare.

In the caudal sections of the hippocampus nNOS neurons were more numerous in the CA1 field and the parasubiculum, showing a pyramidal morphology with dense tufts distributed in the stratum radiatum and stratum lacunosum moleculare.

The superior colliculus contained immunoreactive neurons in all layers. A modest number of nNOS neurons was found in the intermediate gray and white layers and a small number of nNOS neurons was scattered in the deep white layer (Fig. 2D). These immunoreactive neurons were of medium size and multipolar morphology (Fig. 2E).

The cerebellum showed nNOS-immunoreactive stellate and basket cells in the upper and deep portions of the cerebellar molecular layer. The basket terminals around the initial portion of the axons of the mostly non-

immunoreactive Purkinje cells were also immunoreactive (Fig. 2F and G). The granular cell layer contained occasional weakly immunoreactive granular cells. The deep portion of the cerebellum showed immunoreactive varicose fibers and a few nNOS-immunoreactive neurons in the lateral and anterior interposed cerebellar nuclei. Occasional Purkinje cells were stained in the midline of the vermis and in the paraflocculus.

iNOS immunoreactivity. A very small number of scattered iNOS-immunoreactive neurons was found in the cortical areas studied, but iNOS-immunoreactive neurons were a constant feature of the ectal and endal portions of the hippocampal dentate gyrus, where they were distributed in the basal part of the granular cell layer (Fig. 3A). Some cerebellar Purkinje cells were also found to contain iNOS immunoreactivity. All neurons showed the reaction product as small granular deposits in the cytoplasm and the initial portion of main processes.

Nitrotyrosine immunoreactivity. Immunoreactive neurons were found in the cerebral cortex, caudate putamen, islands of Calleja, olfactory tubercle, hippocampus, superior colliculus and cerebellum. The reaction product appeared as granular deposits preferentially distributed in or around the nucleus, but the product was also occasionally found in the cytoplasm of the cell body and/or in the initial portion of the processes (Figs. 3B-H). In the cerebellum, nitrotyrosine immunoreactivity was present in the stellate, basket and Purkinje cells (Figs. 3HI). The glial cells (Fig. 3 J) found in all the cerebral areas studied were also reactive to nitrotyrosine.

15 and 45 min hypobaric hypoxia/24 h reoxygenation

nNOS immunoreactivity. This remained similar to that in control animals after both periods of hypobaric hypoxia (Figs. 4 A-C). The superior colliculus showed a decrease in the number of immunoreactive neurons, which were mainly distributed in the zonal and superficial gray layers. Blood vessels, which retained a normal diameter, were generally surrounded by numerous nNOS-immunoreactive fibers, but no immunoreactivity was found in their endothelial cells (Fig. 5 A-D).

iNOS immunoreactivity. A few iNOS neurons showing the structure and morphology previously described were distributed in the cortical areas (Fig. 5 E), hippocampus (Fig. 5 F and G) and cerebellum (Fig. 5 H and I), the reaction product showing its characteristic granular form in the cytoplasm. Weak immunoreactivity was found in the cerebellar Purkinje cells.

Nitrotyrosine immunoreactivity. This was found in neurons of all areas studied, the reaction product being mainly related to the nuclear area (Fig. 6 A-C).

4 h hypobaric hypoxia/24 h reoxygenation

nNOS immunoreactivity. The number of nNOS-immunoreactive neurons showed an increase in all cortical areas and layers, retaining a morphology similar to those in control animals. Numerous immunoreactive varicose nerve fibers were found in all cortical layers (Fig. 6 D and E), forming a dense network which surrounded the immunoreactive neurons. Blood vessels were slightly dilated in all areas. Small immunoreactive neurons were also seen in all cortical regions, mainly distributed in the upper layer.

The caudate putamen also showed an increase in the number of immunoreactive neurons, these being most numerous just below the ependymal cells of the lateral wall of the lateral ventricle. In addition, nNOS-positive varicose nerve fibers were more prominent in all areas of the caudate putamen (Fig. 7 A and B).

The islands of Calleja in the olfactory tubercle showed intense immunoreactivity in the granular cells that surrounded dilated blood vessels. The nitrergic plexus of the molecular, pyramidal and polymorph layers of olfactory tubercle showed immunoreactive fibers and neurons (Fig. 7 C and D).

The hippocampus also showed increased nNOS immunoreactivity in all regions of Ammon's horn, in the ectal and endal areas of the dentate gyrus and in the hilar region. In general, the processes of the immunoreactive neurons were well developed and showed a varicose structure (Fig. 7 E and F). Dilated blood vessels were also found.

The superior colliculus showed immunoreactive neurons distributed in all layers, which contained numerous dilated blood vessels, giving a spongy appearance to the structure (Fig. 8 A). The dilated blood vessels appeared to stretch the surrounding neural parenchyma, changing the morphology of the many nNOS-immunoreactive neurons from a multipolar to a fusiform shape.

The cerebellum showed a more prominent nitrergic plexus in the molecular layer, where occasional immunoreactive apical processes of Purkinje cells were also seen. The stellate cells, basket cells and basket terminals surrounding the Purkinje cell bodies were also immunoreactive. The granular cells also showed increased immunoreactivity (Fig. 8 B - D).

iNOS immunoreactivity. This was more prominent in all cerebral areas studied. Numerous iNOS neurons were found in the cerebral cortex, specifically as large pyramidal neurons in layers IV-V or small neurons in layer VI of the parietal and temporal regions (Fig. 8 E and D). In the hippocampus immunoreactive pyramidal neurons were also found in the pyramidal layer of the subiculum, and the CA1, CA2 and CA3 fields of Ammon's horn (Fig. 8 F). Immunoreactive multipolar neurons were also distributed in the basal layer of the dentate gyrus, just below the numerous immunopositive granular cells that were also seen in that region. The superior colliculus contained iNOS-immunoreactive neurons in all layers (Fig. 8 G) and some iNOS-immunoreactive Purkinje cells were found in the cerebellum (Figs. 8 H).

Nitrotyrosine immunoreactivity. Numerous nitrotyrosine-immunoreactive neurons were found in the cerebral cortex, olfactory tubercle, superior colliculus and cerebellum (Figs 8 A-F). These showed the reaction product around the nucleus, but reaction product was now also more prominent in the cytoplasm. In the cerebellum, the stellate, basket and Purkinje cells were immunoreactive.

8 h hypobaric hypoxia/24 h reoxygenation

nNOS immunoreactivity. The cerebral cortex showed a general decrease in nNOS immunoreactivity, all areas now acquiring a spongy appearance due to the intensely dilated blood vessels. These blood vessels separated the neural parenchyma into narrow elongated portions which showed an immunopositive network formed by a small number of fusiform or elongated immunoreactive neurons with their sparse processes and collaterals (Fig. 10 A and B). The nitrergic plexus was less prominent in all cortical regions and layers.

The caudate putamen also showed a small number of immunoreactive neurons and nerve fibers forming a nitrergic plexus (Fig. 10 C). The islands of Calleja contained numerous, intensely immunoreactive granular cells surrounding dilated blood vessels (Fig. 10 D).

A few immunoreactive neurons with short processes and collaterals were found in all hippocampal areas (Fig. 10 E). Some pyramidal cells with well-stained cell bodies and processes were occasionally found in CA1 (Fig. 10 F). The blood vessels were also dilated, forming a dense vascular network pervading the hippocampal parenchyma, the ectal and endal regions of the dentate gyrus showing a particularly spongy structure.

Immunoreactivity to nNOS had decreased in all layers of the superior colliculus, where immunoreactive neurons and processes were seen in the compressed bands of parenchyma resulting from the intense dilatation of the blood vessels that gave rise to the characteristic spongy appearance (Fig. 11 A-B).

A similar appearance was found in the cerebellum, where dilated blood vessels crossed the molecular and granular cell layers. Some Purkinje cells showed immunoreactive apical processes and perikarya, which were surrounded by immunoreactive basket terminals (Fig. 11 C-F).

iNOS immunoreactivity. Some iNOS-immunoreactive neurons were scattered throughout the cortical areas, but in layers IV-V of the parietal and temporal cortex these neurons were pyramidal (Fig. 11 G). The hippocampus (Fig. 11 H and I) showed immunoreactive neurons with well-developed immunoreactive processes distributed in different areas of Ammon's horn and among the granular cells of the dentate gyrus. The Purkinje cells in the cerebellum were also stained (Fig. 11 J).

Nitrotyrosine immunoreactivity. All the regions examined contained nitrotyrosine-immunoreactive neurons. Cerebellar Purkinje cells and neurons of the cerebellar nuclei were especially prominent (Fig. 12 A-E).

Blood vessels

In general, the blood vessels of the cerebral cortex showed an intense vasodilatation after exposure to hypobaric hypoxia for 4 or 8 h, followed by 24 h of reoxygenation. This gave rise to the separation of the neuronal parenchyma in narrow, elongated portions in which the stretched nNOS-immunoreactive neurons and their processes appeared to surround the blood vessels as a dense nitrergic plexus (Fig. 13 A).

In particular, the nucleus circularis of the magnocellular accessory group in the anteromedial preoptic area of the hypothalamus, which forms part of the chiasmatic perivascular neurosecretory system, showed nNOS-immunoreactive neurons and processes surrounding the dilated blood vessels after 4 h of hypoxia and 24 h of reoxygenation (Fig. 13 B). After 8 h of hypoxia and 24 h of reoxygenation the dilatation of the blood vessels was the main characteristic, the number of nNOS-immunoreactive neurons surrounding these blood vessels having decreased (Fig. 13 C). At this stages the numerous dilated blood vessels showed nitrotyrosine immunoreactivity in the endothelial cells (Fig. 13 D-E).

L-NAME administration

L-NAME administration did not produce any general changes in neuronal structures, which retained showed a normal appearance in the L-NAME control group.

nNOS immunoreactivity. As in control animals, the cerebral cortex contained nNOS immunoreactive neurons distributed in all layers within a nitrergic plexus (Fig 14 A). The caudate putamen and the islands of Calleja also showed a similar distribution and intensity of immunoreactivity to that seen in controls (Fig. 14 B).

The morphology, intensity of immunoreaction and distribution of nNOS neurons and fibers of the hippocampus (Fig. 14 C and D), cerebellum (Fig. 14 A) and cerebellar nuclei (Fig. 14 B) were also similar to those found in control animals not subjected to hypobaric hypoxia.

iNOS immunoreactivity. The cortex, hippocampus and cerebellum contained iNOS-immunoreactive neurons. The frontal, parietal and temporal cortex showed a small number of immunostained pyramidal cells (Figs. 15 C and D). Some iNOS neurons were also found in the basal portion of the unreactive granular cell layer of the hippocampal dentate gyrus (Fig 15 E).

Nitrotyrosine immunoreactivity. Neurons that were immunoreactive to nitrotyrosine were found in all areas studied, but these were fewer than in animals exposed to the same periods of hypobaric hypoxia and reoxygenation without L-NAME treatment (Fig. 15 F - H).

Western blotting

Cerebral cortical and cerebellar supernatants from control brains and those submitted to 4 and 8 h of hypobaric hypoxia followed by 0 or 24 h of reoxygenation were analyzed by Western blotting using the antisera against whole rat recombinant nNOS, C-terminal peptide of murine iNOS and nitrotyrosine. The nNOS antibody reacted strongly with nNOS, giving a band at 153 kDa. The iNOS antibody give a band at 135 kDa and nitrotyrosine immunoreactivity was shown in two or three bands running between 53 and 23 kDa.

nNOS. The nNOS protein band in the cortex decreased with respect to control animals after 4 and 8 h of hypobaric hypoxia followed by 0 or 24 h of reoxygenation (Fig. 16 A and B). The nNOS protein band in the cerebellum increased after 4 h of hypobaric hypoxia without reoxygenation (Fig. 16 A) but was similar to that of controls after 24 h of reoxygenation (Fig. 16 B). After 8 h of hypobaric hypoxia with 0 or 24 h of reoxygenation the cerebellar nNOS band was similar to or slightly decreased with respect to controls (Fig. 16 C and D).

iNOS. The iNOS protein band in the cortex showed some increase after 4 and 8 h of hypobaric hypoxia followed by 0 or 24 h of reoxygenation, the increase being more marked after reoxygenation (Fig. 17 A and B). The cerebellar iNOS band was maintained after 4 h of hypobaric hypoxia without reoxygenation, decreasing after 8 h of hypobaric hypoxia. A slight decrease in the cerebellar iNOS band was seen after 4 h of hypobaric hypoxia and 24 h of reoxygenation (Fig. 17 C and D).

Nitrotyrosine. Nitrotyrosine immunoreactivity was found in two or three principal protein bands among a considerable number of faint bands. Two main bands of 53 kDa and 23 kDa were found in the cortex and cerebellum. These were little changed in the cortex after 4 and 8 h of hypobaric hypoxia without reoxygenation (Fig. 18 A), but decreased after 24 h of reoxygenation (Fig 18 B). In the cerebellum, both main bands after 4 and 8 h of hypobaric hypoxia with or without reoxygenation (Fig. 18 C and D).

Discussion

These results represent the first extensive immunocytochemical study of the expression of nNOS and iNOS isoforms and the formation of protein nitrotyrosine in the CNS of rats exposed for short periods (15 min to 8 h) to hypobaric hypoxia simulating an altitude of 30,000 ft. (9144 m, 230.4 Torr). They form the basis for a preliminary attempt to assess the role of nNOS, iNOS and protein nitration in the physiological or pathophysiological neural responses to hypobaric hypoxia.

The cerebral cortex, caudate putamen, hippocampus, superior colliculus and cerebellum were studied by immunocytochemistry. The nucleus circularis of the hypothalamus and the islands of Calleja were also studied as two specific types of vascular nuclei, respectively related to the hypothalamic chiasmatic perivascular neurosecretory system and the control of vascular flow to the basal forebrain. The cerebral cortex and cerebellum were analyzed by Western blotting, which reflects overall changes in the concentrations of the studied components in the many different cell types present in these areas and is thus incapable of demonstrating changes confined to minority cell populations.

In general, there is a decreased immunocytochemical expression of nNOS in most cerebral areas by 8 h of hypobaric hypoxia, counterbalanced by an increased expression of iNOS. Protein nitrotyrosine reflecting nitration due principally to peroxynitrite formation did not show significant global modifications immediately after 4 or 8 h of hypoxia, but the distribution of the reaction product ran in parallel with the nNOS and iNOS immunoreactivities in the areas studied.

Some parallels with these results have been observed by us in previous immunocytochemical studies. Thus iNOS appeared in cortical neurons of rat forebrain slices subjected to 3 h of oxygen and glucose deprivation *in vitro*, at which time nNOS expression had decreased (Moro et al., 1998). In a perfusion model of global cerebral ischemia, nNOS immunostaining increased in cerebellar stellate and basket cells and iNOS appeared in Purkinje cells after 2-4 h of reperfusion following 30 min of oxygen and glucose deprivation, accompanied by an increase and morphological changes in the intracellular distribution of protein nitrotyrosine (Rodrigo et al., 2000). The cerebral cortex of aging rats shows iNOS expression by pyramidal neurons of layers IV-V (Uttenthal et al., 1998) and this is also observed in the cerebral cortex from brains subjected to global ischemia (Alonso et al., 2001, in preparation).

Western blotting shows that the overall expression of nNOS in the cerebral cortex tends to decrease after 4 or 8 h of hypobaric hypoxia, while iNOS expression shows an increase that is particularly apparent after 24 h of reoxygenation. While iNOS immunoreactivity increases in cerebellar Purkinje cells after 4-8 h of hypobaric hypoxia, overall iNOS expression showed little change or tended to decrease. Nitrotyrosine immunoreactivity was present in two main protein bands of 53 and 23 kDa respectively, which decreased in the cortex and increased in the cerebellum after 4-8 h of hypobaric hypoxia.

Prabhakar et al. (1996) have reported immunocytochemical changes in nNOS in the cerebellum of adult rats exposed to hypobaric hypoxia (304 mm Hg/0.4 atm.) for up to 24 h, showing that the increase in nNOS immunoreactivity depended on the time of exposure. Our results suggest that not only exposure time but also the reoxygenation period may influence the magnitude of changes in the expression of the NOS isoforms studied.

Histological changes consequent on hypoxia have previously been described in the hippocampus, which is the cerebral area most sensitive to hypoxia and the seat of various cognitive and memory functions (Ando et al., 1987) These changes involved damage to the pyramidal cells of the CA1 field of Ammon's horn and the dentate gyrus (Brierly, 1976; Jensen et al., 1991; Katoh et al., 1992; Kirino, 1982; Shukitt-Hale et al., 1994, 1996). Other cerebral

regions such as the cerebral cortex, striatum, thalamus, cerebellum and amygdala were also described as being affected by neuronal damage following hypoxia (Brierly, 1976; Katoh et al., 1992; Shukitt-Hale et al., 1994, 1996).

In the present study we have not only found that the immunocytochemical expression of nNOS, iNOS and nitrotyrosine underwent changes in the cortex, striatum, islands of Calleja, hippocampus, superior colliculus and cerebellum, but also that these cerebral areas show numerous dilated blood vessels after hypobaric hypoxia. These dilated vessels displace the neural parenchyma, giving rise to a markedly spongy appearance of the nervous tissue. This dramatic vasodilatation must directly affect the cerebral blood flow and influence the aerobic mechanisms that sustain the Krebs cycle and the cellular respiratory chain (Bolaños et al., 1994), as well as synaptic neurotransmission (Cavaletti and Tredici 1991).

It is possible that this extreme vasodilatation, by displacing and stretching the neural parenchyma with its many neural elements, may produce transient or even permanent changes in neuronal connections and relations, and thus contribute to some of the various clinical symptoms described by Bahrke and Shukitt-Hale (1993).

While the expression of iNOS in cerebellar neurons can be induced by LPS and interferon-γ (Minc-Golomb et al., 1994, 1995), the iNOS gene promoter can also be activated by a hypoxia-responsive element (Melillo et al., 1995). We have recently demonstrated that oxygen and glucose deprivation induced iNOS expression in rat forebrain slices, as assessed by the detection of iNOS mRNA and protein. A six-fold increase in iNOS mRNA was observed at 180 minutes and the time course of iNOS mRNA expression was in agreement with the temporal profile of iNOS (calcium independent) enzymatic activity. Immunocytochemistry revealed that iNOS was expressed in neurons, astrocytes and microglial cells (Moro et al., 1998).

The rise in intracellular calcium levels, which activates the constitutive isoform nNOS, appears to play an integral role in the production of ischemic and hypoxic cell damage. *In vivo* calcium accumulates in the cells that are selectively vulnerable to ischemia (Deshpande and Wierloch, 1985; Deshpande et al., 1987; Gibson et al., 1988; Jensen et al., 1991) and mediates cellular damage (Griffiths et al., 1982, 1983; Simon et al., 1984a,b; Van Reempts et al., 1984). Calcium antagonists or glutamate-receptor blocking agents ameliorate ischemic damage (Newberg et al., 1984; Deshpande et al., 1987, Vibulsresth et al., 1987; Buisson et al., 1993) and the omission of calcium from the medium diminishes the biochemical (Gibson and Mykytyn, 1987; Harvey et al., 1983) and electrophysiological (Kass and Lipton, 1982) effects of an anoxic preincubation. The elevated intracellular free calcium levels could also disrupt cellular metabolic activity and eventually cause neuronal death (Mitani et al., 1990).

The activation of nNOS by elevated intracellular calcium and the induction of iNOS expression by hypoxia leads to an increase in the biosynthesis of NO. NO decays in solution with a half-life of 5-10 seconds, whereas the citrulline that is formed in equimolar amounts remains in the cells and can be reconverted into L-arginine to provide further substrate for NO synthesis (Bredt and Snyder, 1990; Knowles et al., 1989; Moncada et al., 1991). The increased generation of NO that takes place during hypoxia, at first due to nNOS activation by calcium and subsequently sustained by induction of the highly active iNOS, produces a strong vasodilatation that would appear to permit a compensatory increase in cerebral blood flow. However, the dramatic extent of the vasodilatation observed may also result in the potentially damaging structural neural changes described above.

NO reacts with superoxide radicals to form peroxynitrite, a potent oxidant that is capable of nitrating tyrosyl residues in tissue proteins (Beckman et al., 1994; Beckman, 1996; Szabó, 1996). The nitrotyrosine formed thus acts as a direct marker of peroxynitrite generation over a period that is determined by the turnover of the protein in question. The existence of possible denitration mechanisms (Kamisaki et al., 1998) will also influence the amount of nitrotyrosine immunoreactivity observed. As peroxynitrite is formed by the combination of NO with superoxide, nitrotyrosine also acts as an indirect marker of NO production and its possible neurotoxic effect (Lipton et al., 1993; Zhang and Snyder, 1995). Our immunocytochemical results suggest a certain parallelism of nitrotyrosine formation with the expression of nNOS and iNOS during hypobaric hypoxia. In normal conditions the reaction product is usually found in the cell nucleus and perinuclear areas, but not in the cell processes (Uttenthal et al., 1998; Bolan et al., 2000; Rodrigo et al., 2000). However, after acute ischemia or hypoxia the reaction product appears in the cytoplasm and processes (Rodrigo et al., 2000). This anatomical translocation is more remarkable than any quantitative changes in overall protein nitration as determined by Western blotting.

A moderate amount of protein nitrotyrosine is present in some cells throughout life and is considered to be a feature of normal physiology (Uttenthal et al., 1998). However, large amounts of nitrotyrosine have been demonstrated in some inflammatory disorders, atherosclerotic lesions and neurodegenerative diseases (Beckman et al., 1994; Bagasra et al., 1995; Beckman, 1996; Eiserich et al., 1998), in the brains of aging rats (Uttenthal et al., 1998) and brains submitted to global ischemia (Rodrigo et al., 2000; Alonso et al., 2001). Different nitrated protein bands may be detected under different circumstances. A nitrated 53-54 kDa band is commonly seen in rat brain extracts together with nitrated bands of lower molecular mass, and in the present experiments the major nitrated bands were found at 53 and 23 kDa. How protein nitration and other types of oxidative damage caused by peroxynitrite and other reactive nitrogen species cause neuronal death is not understood in detail, but it has been postulated that a neurotoxic effect might be produced by the nitration of mitochondrial respiratory chain enzymes (Bolaños et al., 1994), or in general, that nitration of tyrosine residues alters the conformation and functional activity of proteins, as well as their susceptibility to digestion (Castro et al 1994; Berlett et al., 1996; Crow et al., 1994). The nitration of tyrosine residues may also decrease the effectiveness of

certain proteins as substrates for tyrosine kinases (Martin et al., 1990, Crow et al., 1994; Wink et al., 1993a,b). These neurotoxic effects of the NO-peroxynitrite-nitrotyrosine pathway may directly participate, in an irreversible or only slowly reversible manner, in the interruption of cellular signaling processes.

The administration of L-NAME for five days prior to submitting the rats to hypobaric hypoxia blocked the appearance of the marked cerebral vasodilatation, indicating that this effect is directly related to the activity of NOS isoforms synthesizing NO, which acts as a potent physiological vasodilator. L-NAME administration also attenuated many of the other immunocytochemical changes observed in the rats submitted to 8 h of hypobaric hypoxia, so that sections from these brains were generally similar in appearance to those from control rats. Exceptions to this generalization were that iNOS still appeared in some cortical neurons and that nitrotyrosine immunoreactivity was attenuated to below control levels.

Finally, it is possible that the anatomical changes observed after 4-8 h of hypobaric hypoxia are reversible after a longer recovery period and may be related to some of changes in neuropsychological functions observed after human exposure to high altitude.

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